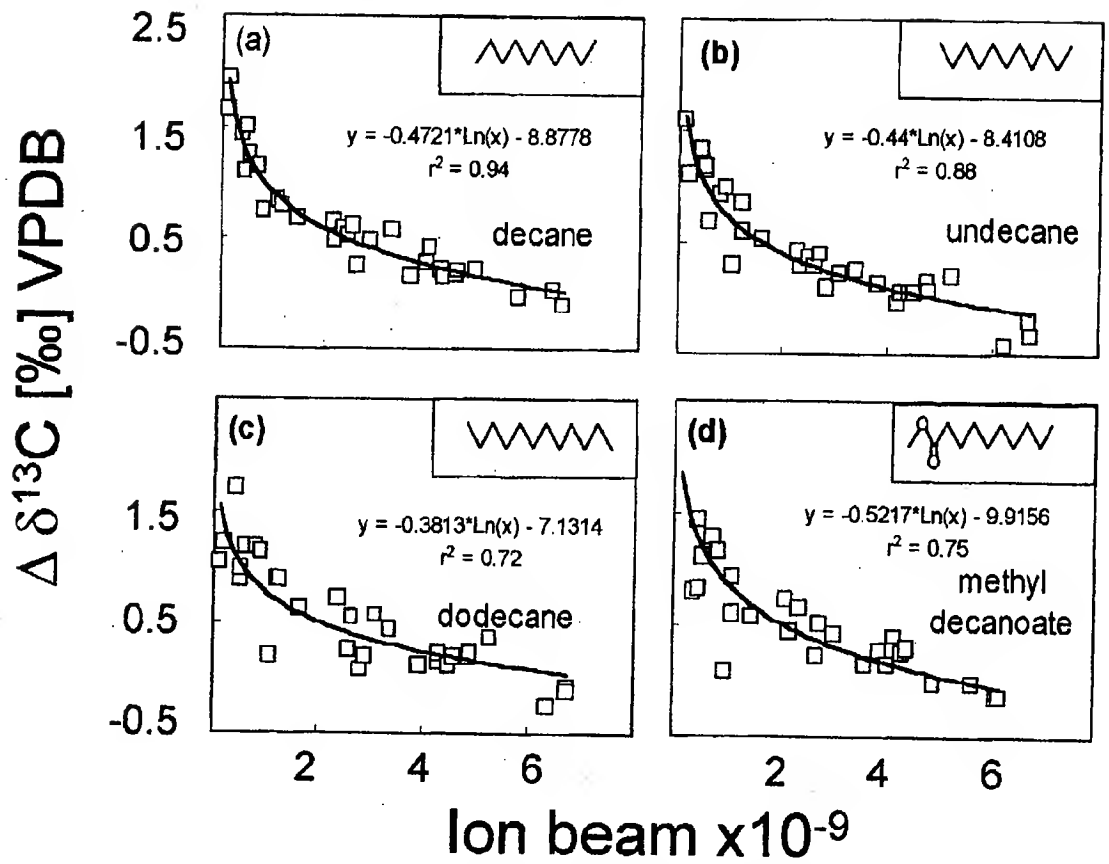

Figure 3. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta\ \delta^{13}C_{DIC}$) for decane, undecane, dodecane and methyl decanoate. The upper right corner of each graph shows the structure of the analyte.

produced $\delta^{13}C$ values that were signal dependent. Our initial investigations showed that although the $\delta^{13}C$ values produced for the VG mix were approximately non-signal dependent to ±0.3‰ for the ion beam intensities between 1×10^{-9} and 1×10^{-8} A, the sample accuracy and precision soon degraded significantly between 1×10^{-10} and 1×10^{-9} A. A series of injections was undertaken to produce I^{44} intensities of between 1×10^{-10} and 8×10^{-8} A. Figure 3 illustrates that the $\delta^{13}C$ values produced for decane, undecane, dodecane and methyl decanoate are exponentially dependent on the I^{44} intensity. The delta value (Δ) plotted represents the difference between the theoretical value given by Micromass UK Ltd. and the values recorded by the IRMS. All four compounds showed a positive Δ of between 1.6 and 2‰ when the ion beam signal was smaller than 5×10^{-10} A. This enrichment decreased with increasing I^{44} intensity. Figure 3 demonstrates that the theoretical $\delta^{13}C$ value is approached when the I^{44} intensity is between 5.5×10^{-9} and 7×10^{-9} A. This is because the $\delta^{13}C$ value of the reference gas used in the IRMS was cross-calibrated using VG mix at I^{44} intensities of 6×10^{-9} to 6.5×10^{-9} A. The relative molecular masses of these compounds varied from 142 to 172 Da. The logarithmic regression observed in Fig. 3 does not appear to be mass dependent. The MSD successfully identified all compounds plotted in Fig. 3 to at least a 93% confidence level.

Bioremediation of phenol is currently ongoing within the EERC.[18] Phenol was therefore selected to study the effect of signal intensity on the $\delta^{13}C$ value recorded for a smaller molecule. Sample sizes analysed were again varied to produce I^{44} intensities of 1×10^{-10}–1×10^{-8} A. Figure 4(a) illustrates that the $\delta^{13}C$ values produced were again

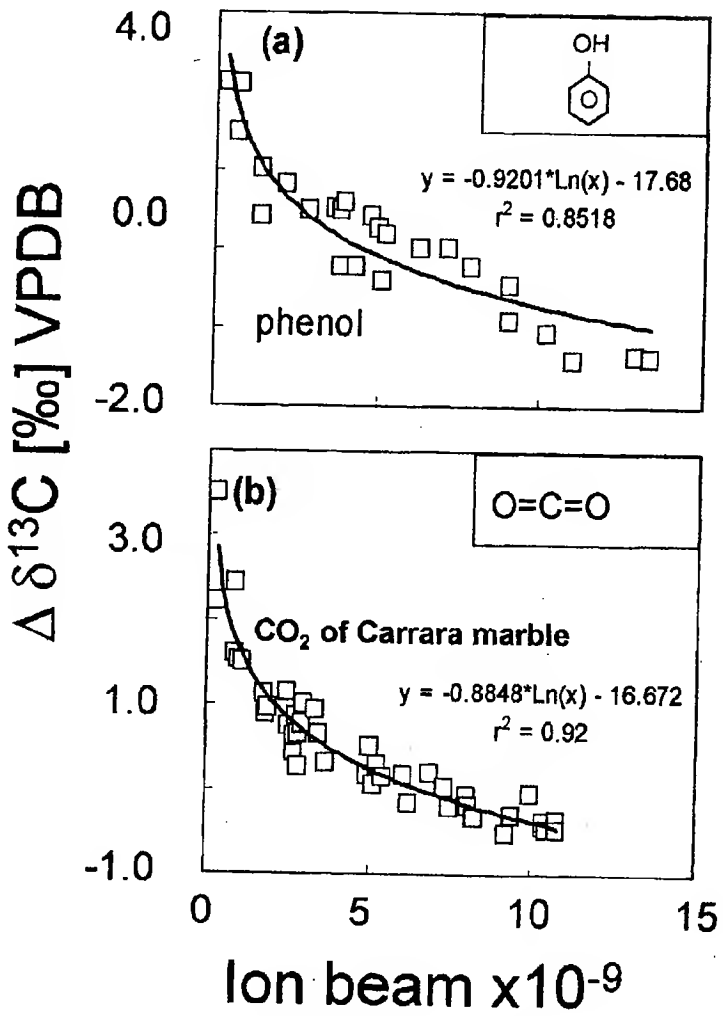

Figure 4. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta\ \delta^{13}C_{DIC}$) for phenol and CO_2 from Carrara marble. The upper right corner of each graph shows the structure of the analyte.

signal dependent. The $\delta^{13}C$ value becomes more enriched with decreasing sample size, reaching a maximum enrichment of 1.2‰ at an I^{44} intensity of 7×10^{-10} A. The theoretical $\delta^{13}C$ value of phenol (−28.5‰) is approached when the I^{44} intensity is 6.8×10^{-9} A.

CO_2 standards

Our aim was also to investigate the role of the combustion tube in the signal dependence trend observed. A series of CO_2 analyses derived from the acidification of Carrara marble were performed. Injecting CO_2 directly onto the column with identical operational conditions for the combustion interface obviously negates the necessity of CuO in the combustion interface to oxidise the carbon present in the sample. Figure 4(b) shows the $\delta^{13}C$ value plotted against ion beam size. The Carrarra marble standard shows a maximum enrichment of 2.5‰ for an ion beam size of 3×10^{-10} A. The true value of the standard (2.4‰) is approached for an ion beam size of 6.5×10^{-9} A.

DISCUSSION

This investigation outlines the successful coupling of two independent detectors, an MSD and an IRMS to a GC. When using standard gas chromatography/mass spectrometric analysis it is important to optimise the detector to enable quantification and qualification of the smallest possible sample size. The IRMS used in our system has been optimised to operate at a high sensitivity. This now enables the quantification, qualification and $\delta^{13}C$ values of compounds to be recorded from one sample analysis.

After compromising the signal independence by tuning the IRMS for higher sensitivity, a logarithmic relationship occurs between the $\delta^{13}C$ value and the I^{44} intensity for all four compounds in Fig. 3. By manipulating the equation best describing the points ($y = m^* \ln(x) + c$) in Fig. 3, Eqn. 2 is derived:

$$\delta^{13}C_{true} = \delta^{13}C_{actual} - ((m^* \ln(I^{44})) + c) \qquad (2)$$

We were then able to correct the $\delta^{13}C$ values obtained for any I^{44} between 2×10^{-10} and 7×10^{-9} A. Using this correction equation, the compounds in Fig. 3 produced $\delta^{13}C$ values to an accuracy of <0.01‰ and a precision of between 0.13 and 0.23‰. Corrected phenol $\delta^{13}C$ values were also calculated using Eqn. 2. Over two orders of magnitude the accuracy using this technique is <0.01‰ with a precision of 0.3‰. This precision is not particularly good. This could be an artefact of sample degradation over time. The results were obtained over two days and phenol has been shown to degrade as a function of time in ethyl acetate.[20]

The $\delta^{13}C$ values of the organic compounds investigated all display a signal dependence relative to I^{44} intensity. A possible source of instrumental error could be the combustion interface.[8] To elucidate whether this may the case CO_2 was injected directly onto the column. The Carrara marble data showed the same logarithmic signal dependence shown by compounds that were injected on-column as liquids and combusted on the CuO interface. The $\delta^{13}C$ values could therefore also be corrected by Eqn. 2. When the correction was performed, the $\delta^{13}C$ values obtained were accurate to 0.01‰ with a precision of 0.25‰ from 2.2×10^{-10} to 1.1×10^{-8} A. The IRMS does have the potential for very good internal precision. Table 2 shows the $\delta^{13}C$ values for analysis of CO_2 from decane over a range of sample sizes. Before adjusting the $\delta^{13}C$ values for signal size, the internal precision of the instrument is 0.53‰ for a range of I^{44} between 1.2×10^{-10} to 6.6×10^{-9} A. After correction, the average of the calculated value exactly matches the theoretical value of −28.61‰, while the precision decreases to 0.13‰.

Table 2. Measured and corrected $\delta^{13}C$ values of decane at variable ^{44}I beam intensities. The average of the latter shows a significant increase in precision and coincides with the theoretical value (high accuracy)

Ion^{44} beam	$\delta^{13}C$ measured	$\delta^{13}C$ corrected
1.19E-10	−26.96	−28.87
1.61E-10	−26.68	−28.45
4.00E-10	−27.18	−28.52
4.76E-10	−27.51	−28.77
4.82E-10	−27.11	−28.36
5.49E-10	−27.36	−28.55
7.47E-10	−27.47	−28.51
8.46E-10	−27.88	−28.86
1.11E-09	−27.78	−28.64
1.17E-09	−27.82	−28.65
1.48E-09	−27.93	−28.65
2.17E-09	−27.97	−28.51
2.18E-09	−28.13	−28.67
2.36E-09	−28.02	−28.52
2.43E-09	−28.09	−28.58
2.53E-09	−28.00	−28.47
2.64E-09	−28.36	−28.81
2.88E-09	−28.14	−28.55
3.27E-09	−28.04	−28.39
3.66E-09	−28.46	−28.75
3.95E-09	−28.34	−28.60
4.01E-09	−28.19	−28.44
4.23E-09	−28.40	−28.62
4.28E-09	−28.46	−28.68
4.52E-09	−28.44	−28.63
4.55E-09	−28.41	−28.60
4.88E-09	−28.40	−28.56
5.70E-09	−28.65	−28.73
6.38E-09	−28.58	−28.61
6.57E-09	−28.73	−28.75
Average	−27.98	−28.61
1σ*	0.53	0.13

* Error to one standard deviation.

As can be seen from the equations in Figs 3 and 4, the trends for the $\delta^{13}C$ values of the compounds in the VG mix, phenol and CO_2 are similar. Figure 5 illustrates all data points recorded ($\Delta = \delta^{13}C_{actual} - \delta^{13}C_{true}$) plotted as a function of the I^{44} intensity. The r^2 value in the equation describing the signal dependence is 0.72. This suggests that an overall correction factor could be used for all data to enhance $\delta^{13}C$ accuracy and precision over the I^{44} ranges of 2×10^{-10} to 1×10^{-8} A. However, we have found from the data presented that compound specific correction achieves better results.

The data presented here clearly show that the combustion interface performance can not account for the trends outlined in this paper. The water trap, if properly maintained, is of a reliable design and is also an unlikely source of any routine error. With decreasing sample size, any air in the system, and in particular nitrogen, will become a larger proportion of the total sample size. The possible contribution of any incompletely oxidised N_2 in the form of

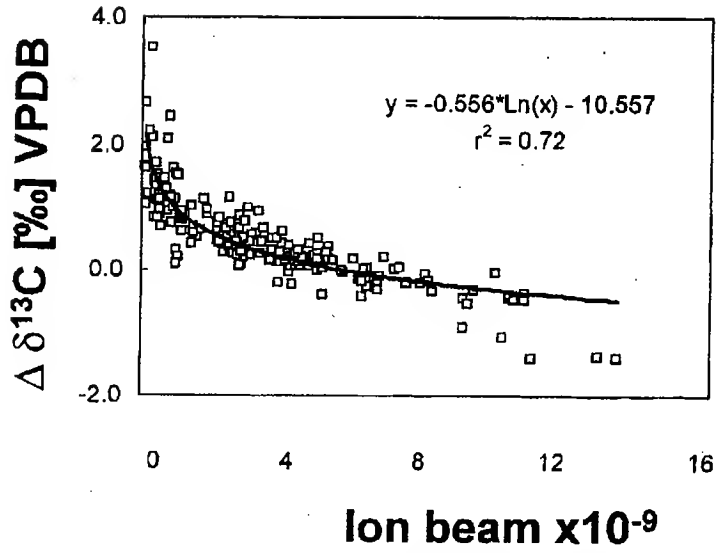

Figure 5. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta\ \delta^{13}C_{DIC}$) for decane, undecane, dodecane and methyl decanoate, phenol and CO_2 from Carrara marble plotted in one graph.

N_2O was therefore of concern. However, any addition of ionised N_2O to the I^{44} beam would produce depleted $\delta^{13}C$ values with decreasing I^{44} intensity, the opposite of the trend observed. It is interesting to note that the application of a logarithmic correction to sample size dependant $\delta^{13}C$ values has also been reported for duel inlet measurements.[21] However, the major difference between continuous flow and duel inlet sample introduction makes the previously published explanation inappropriate. One possible explanation for the signal dependence may be mass diffusion in the IRMS source. There is a flow of CO_2 and helium (He) into the ion source. As the ratio of CO_2 to He changes (i.e. decreasing beam I^{44} strength), there may be an increasing potential to accentuate the diffusional fractionation of mass 44, 45 and 46 and therefore cause variation in residence times of these ions within the source. Further work is needed to elucidate the exact nature of the observed phenomenon.

CONCLUSIONS

A GC has been coupled successfully with two detectors, an MSD and an IRMS. The nature of the flow splits in the instrumental coupling make it desirable to achieve a higher degree of sensitivity than may normally be required when using a routine GC/IRMS. The signal dependent trend observed is similar for all the compounds studied in this paper. However, the signal dependence that occurs from a high sensitivity source tuning can be corrected for. The results indicate that a general correction equation could be used to correct $\delta^{13}C$ values for I^{44} intensity. However, where possible, compound specific correction provides the best accuracy and precision. This allows routine continuous flow analysis using a GC/MSD/IRMS system to produce very accurate results to a precision approaching 0.1‰ over two orders of I^{44} magnitude.

Acknowledgements

M. Carey, N. Ogle and J. Hamilton are thanked for their technical assistance. This work was supported by grants from the QUESTOR Industrial Board, Queen's University Belfast, and EPSRC grants L85183 and M26374.

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GDC01133

Use of gas chromatography–combustion–isotope ratio mass spectrometry in nutrition and metabolic research

Wolfram Meier-Augenstein

Linking gas chromatography via an on-line combustion interface to isotope ratio mass spectrometry has opened the door to high-precision compound-specific isotope analysis. For this reason, gas chromatography–combustion–isotope ratio mass spectrometry is now increasingly employed in metabolic and nutritional research because it offers a reliable and risk-free alternative to the use of radioactive tracers. Curr Opin Clin Nutr Metab Care 2:000–000.

Correspondence to Wolfram Meier-Augenstein, CChem MRSC, Senior Research Fellow, University of Dundee, Department of Anatomy and Physiology, OMS, Small's Wynd, Dundee, DD1 4HN, UK
Tel: +44 0 1382 345124; fax: +44 0 1382 345514; e-mail: w.meieraugenstein@dundee.ac.uk

Current Opinion in Clinical Nutrition and Metabolic Care 1999, 2:000–000

Abbreviations

APE	atom% excess
BCAA	branched-chain amino acids
GC–C–IRMS	gas chromatography–combustion–isotope ratio mass spectrometry
CSIA	compound-specific isotope analysis
GC–MS	gas chromatography–mass spectrometry
IRMS	isotope ratio mass spectrometry
SIM	selected ion monitoring mode

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Introduction

One of the reasons for the increasing appeal of gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) is the fact that, as a result of this technology, stable isotope labelled tracers have become a viable alternative to radioactive tracers, a fact that is of particular relevance when dealing with paediatric patients. With the exception of deuterium (^{2}H) when used in high concentrations, heavier stable isotopes such as ^{13}C, ^{15}N and ^{18}O do not cause any adverse physiological effects, even at high enrichment levels [1]. Isotope ratio mass spectrometry (IRMS) is an analytical mass spectroscopic technology, for it provides quantitative information rather than structural information on a given compound. The application of high-precision compound-specific isotope analysis (CSIA) in nutrition and metabolic research can be roughly divided into two areas. One area of application is concerned with studies to measure the rate of intermediary metabolism, the other aims to trace metabolic pathways using labelled precursor compounds at high enrichment levels to improve detection limits. This article aims to present an overview of the work in these areas during the past 4 years. Because this is the first time that GC–C–IRMS is presented in this forum, some emphasis is also placed on the methodological aspects and constraints of this hyphenated technique.

Practical issues concerning gas chromatography–combustion–isotope ratio mass spectrometry

In contrast to organic mass spectrometers that yield structural information by scanning a mass range over several hundred Daltons for characteristic fragment ions, IRMS instruments achieve a highly accurate and precise measurement of isotopic abundance at the expense of the flexibility of scanning mass spectrometers (Table 1). Because gas chromatography–mass spectrometry (GC–MS) can be used to measure stable isotope enrichment, the question arises as to why one should embrace GC–C–IRMS. Scanning mass spectrometers use a single detector and therefore cannot simultaneously detect particular isotope pairs for isotope ratio measurement. For isotope ratio measurement, the mass spectrometer is best operated in selected ion monitoring mode (SIM) to optimize the sensitivity to selected masses. Even in SIM mode, the limited accuracy and precision of such isotope ratio measurements impose a minimum working enrichment for ^{13}C and ^{15}N of at least 0.5 atom% excess (APE) [2,3]. In other words, organic mass spectrometry cannot

provide reliable quantitative information in cases in which a low turnover or low rate of incorporation results in isotopic enrichment of less than 0.5 APE.

In contrast, GC–C–IRMS can measure isotopic composition at low enrichment and natural abundance levels. This means that minute variations in very small amounts of the heavier isotope are detected in the presence of large amounts of the lighter isotope. Because the small variations of the heavier isotope habitually measured by IRMS are of the order of −0.07 to +1.09 APE, the δ-notation in units of per mil (‰) has been adopted to report changes in isotopic abundance as a per mil deviation compared with a designated isotopic standard:

$$\delta_s = ([R_s - R_{std}]/R_{std}) \times 1000\ [‰]$$

where R_s is the measured isotope ratio for the sample and R_{std} is the measured isotope ratio for the standard. To give a convenient rule-of-thumb approximation, in the δ-notation, a ^{13}C enrichment in the range of −0.033 to +0.0549 APE corresponds to a $\delta\ ^{13}C$ value range of −30‰ to +50‰. A change of +1‰ is approximately equivalent to a change of 0.001 APE and 0.0003 APE for ^{13}C and ^{15}N, respectively.

The sensitivity of GC–C–IRMS is such that tracer/tracee (mol/mol) ratios down to 10^{-5} can be reliably detected [4]; in the same review, Brenna *et al.* also provide an in-depth discussion of notations and elementary calculations such as mass balance and pool mixing equations.

As a result of its high sensitivity, GC–C–IRMS depends on careful sample preparation and high-resolution capillary gas chromatography [5••]. Demands on sample size, sample derivatization, quality of gas chromatography separation, interface design and isotopic calibration have been discussed in a number of reviews [2,3,5••,6–8,9••].

High-precision compound-specific isotope analysis of ^{13}C isotopic abundance

The great clinical and scientific potential of ^{13}C tracer techniques for nutritional and metabolic research in paediatric patients has been discussed recently [10••]. Several comparative studies have demonstrated the considerable advantages of GC–C–IRMS in nutritional and metabolic research. Pont *et al.* [11] measured ^{13}C enrichment in the cholesterol of rabbit low-density lipoprotein after the injection of 3 mg of [3,4-^{13}C]cholesterol. They compared the accuracy and precision, detection limits and dynamic range (ranging from −22 to +760) of GC–C–IRMS and GC–MS measurements and found GC–C–IRMS to be more accurate and reproducible, especially at lower enrichment levels. Highly linear calibration curves for ^{13}C enrichment were also reported from a study with branched-chain amino acids (BCAA) [12]. In that study, slopes of 0.98 and 1.04 were observed for enrichment ranges 0–0.14 APE and 0–

Table 1. Typical features and specifications of mass spectrometry systems used for stable isotope analysis

	GC-MS	Elemental analyser– IRMS	GC–C–IRMS
Sample introduction	Injection of liquid (or gaseous) sample matrix on to GC column	Solid (and dry) sample in tin capsules	Injection of liquid (or gaseous) sample matrix on to GC column
Sample separation	Yes, by gas chromatography	No	Yes, by gas chromatography
Sample manipulation before mass spectroscopic analysis	None	Combustion/reduction of sample into CO_2 and N_2 in the elemental analyser	Combustion/reduction of compounds into CO_2 and N_2 in the interface
Interface	Heated transfer capillary directly connected to ion source	Transfer capillary with open split	Capillary, incorporating wide bore combustion/reduction furnaces
Mass analysers	Quadrupole	Magnet	Magnet
Detector	One electron multiplier	Triple Faraday cup collector	Triple Faraday cup collector
Mode of charged mass Detection	SIM, switching between e.g. M^+ and $[M+1]^+$ ($[M+1]^+/M^+$ ratios are calculated on the basis of measured ion current	Simultaneous detection of particles with three adjacent masses, e.g. m/z 44, 45 and 46 for $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$, respectively	Simultaneous detection of particles with three adjacent masses, e.g. m/z 28, 29 and 30 for $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$, respectively
Measureable enrichment range in [APE]	+0.5 − 100[a]	−0.1 − +2.0	−0.1 − +2.0
Sample size requirement	⩽1 pmol	0.1 − 5 μmol[b]	0.1 − 5 nmol[c]
CSIA	Yes, with a precision of 0.05 atom%	No[d]	Yes, with a precision of 0.0002 atom%

[a]Using a multiply labelled tracer (e.g. [$^2H_5$]phenylalanine) and measuring $[M+n]^+/M^+$ ratios (e.g. $[M+5]^+/M^+$), enrichments down to 0.2 APE can be reliably detected.
[b]The required sample size depends on the type of isotope analysis. Analysis of ^{13}C enrichment requires only small amounts of material because of the high abundance of carbon in organic compounds. The opposite is true for the analysis of ^{15}N enrichment, because of the low abundance of nitrogen in organic compounds. Furthermore, for compounds containing only one atom of nitrogen, two molequivalents of compound have to be combusted to generate one molequivalent of N_2.
[c]In addition to the considerations mentioned above, it should be noted that compound-specific isotope analysis (CSIA) by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) requires the injection of 0.1–5 nmol per individual compound to be analysed.
[d]Using elemental analyser–IRMS, CSIA is only possible for off-line isolated and purified compounds. Here, the precision is 0.001 atom%.
APE, Atom% excess; GC–MS, gas chromatography–mass spectrometry; SIM, selected ion monitoring mode.

8 APE, respectively. Parker *et al.* [13] showed that GC–C–IRMS permits the use of low doses of β-[U-^{13}C]carotene (⩽2 mg), which do not perturb endogenous pool sizes of β-carotene or retinol. Employing ^{13}C tracers at low levels of enrichment, reliable data were obtained in studies of the kinetics of glycoprotein neutral sugars [14] and urea [15]. Bunt *et al.* [16•] used [U-^{13}C]glucose as a precursor to obtain data on endogenous surfactant production and turnover by measuring ^{13}C-enrichment of palmitic acid in phosphatidylcholine palmitate. GC–C–IRMS was also used to determine the range of natural isotope abundance of ^{13}C in six serum fatty acids from humans on a controlled diet [17].

Compound-specific isotope analysis of ^{13}C labelled fatty acids

The high sensitivity of GC–C–IRMS has been increasingly exploited in metabolic studies investigating the turnover, incorporation and synthesis processes of fatty acids *in vivo*, which could only previously be investigated either with stable isotope tracers at high enrichment levels using GC–MS, or not at all [18].

GC–C–IRMS, capable of measuring naturally occurring differences in isotope ratios, determined the extent to which neonates and very low birthweight premature infants could synthesize arachidonic acid, which is essential for their growing tissues, from dietary fatty acids. Demmelmair *et al.* [19] showed that in neonates fed on a phenylalanine-free diet, on average 23% of free plasma arachidonic acid on study day 4 originated from infantile linoleic acid conversion. Carnielli *et al.* [20] added linoleic acid and linolenic acid, both ^{13}C-labelled, to the formula diet that was administered continuously for 48 h (birthweight 1.17±0.12 kg; gestational age 28.4±1.3 weeks). The authors demonstrated that both tracers were rapidly incorporated into plasma phospholipids and that their metabolic products including arachidonic acid and docosahexaenoic acid became highly enriched with ^{13}C. The incorporation of ^{13}C-octanoic acid into plasma triglycerides (10% of the enrichment of the diet), noticeably into myristic and palmitic acid, by very low-weight preterm infants was reported earlier by the same group [21].

The majority of ^{13}C tracer studies published have dealt with various aspects of fatty acid biochemistry. The bioequivalence of dietary α-linolenic and docosahexaenoic acid as substrates for brain and retinal n3 fatty acid accretion during the brain growth spurt [22,23•], metabolism of triglycerides [24], transport and turnover of free saturated [25,26,27•] and unsaturated fatty acids [28–30,31•]. While studying the metabolism of ^{13}C-labelled polyunsaturated fatty acids by ^{13}C-nuclear magnetic resonance, using GC–C–IRMS, Cunnane *et al.* [32] found low levels of ^{13}C-labelled γ-linolenic acid in the brain phospholipids of suckling rat pups that could not be detected by ^{13}C-nuclear magnetic resonance.

Unlike GC–MS, in which increasing the amount of label has no general effect on detection limits, in GC–C–IRMS, increasing label enrichment in precursor compounds produces significantly improved detection limits. Taking advantage of this increased traceability, Rhee *et al.* [33] quantified the desaturation of [U^{13}C]18:0 and [U^{13}C]16:0 in whole plasma and lipoprotein of adults after the administration of 30 mg oral doses (<0.5 mg/kg). Su and Brenna [34•] reported the simultaneous measurement of desaturase activities by monitoring the increase in product from the reactions [U^{13}C]18:2n6→[U13C]18:3n6 and [U^{13}C]16:0→[U^{13}C]16:1n7, respectively.

Using [U^{13}C]α-linolenic acid, Sheaff *et al.* [35] were able to demonstrate that high dietary levels of linoleic acid did not depress the conversion of α-linolenate into docosahexaenoate. Three years later, Menard *et al.* [36•] showed that [U^{13}C]α-linolenic acid is not solely a source of docosahexaenoic acid. They found that, owing to a high rate of β-oxidation and carbon recycling, [U^{13}C]α-linolenate was utilized in the de-novo synthesis of cholesterol and palmitate.

Compound-specific isotope analysis of ^{13}C-labelled amino acids

In spite of the wide usage that GC–C–IRMS enjoys in research concerned with fatty acid and surfactant metabolism, most research groups interested in amino acid metabolism and protein turnover still seem to favour traditional GC–MS methods such as SIM in conjunction with poly-deuterated tracers or tracers highly enriched in ^{13}C. This may be because GC–MS methods cannot easily be adapted to suit the particular needs of GC–C–IRMS. As mentioned above, the nature of the derivatization agent can influence the accuracy and precision of CSIA, because it will dilute ^{13}C enrichment and can adversely affect chromatographic separation and the efficiency of the combustion catalyst (cf. Table 2) [5••,7,9••].

Seemingly in response to these difficulties, most groups using GC–C–IRMS to study turnover and synthesis rates of various proteins have adopted a strategy whereby a target amino acid is identified that will act as a marker providing unambiguous and quantitative information about the system under investigation. This strategy included the development of a sample preparation protocol tailor-made for the particular amino acid allowing quick and reliable CSIA.

In this way, L-[^{13}C-1]valine was employed to measure in-vivo secretion rates of very low density lipoprotein-

Table 2. Overview of typical analytical procedures used in metabolic and nutritional research involving stable isotopes and their caveats

Compound class	Derivatization	Typical GC conditions[a]	Caveats
Long-chain alcohols and sterols (e.g. cholesterol)	TMS	CP-Sil 5: 50°C (2 min) to 200°C at 40°/min, 200–320°C at 3°/min	None
Fatty acids	Methyl ester using metanol/BF_3	CP-Sil 8/CP-Sil 19: 120°C (4 min) to 280°C at 4°/min	None
Hydroxy/amino/keto-carboxylic acids	TMS or *t*BDMS	CP-Sil 8: 80°C (5 min) to 150°C at 2°/min, 150–220°C at 3.5°/min; 20°/min to 300°C	TMS: multiple derivatives for same compound[b] *t*BDMS: excessive carbon load[c]
Hydroxy/amino.keto-carboxylic acids	Ethylchloroformates	CP-Sil 24: 60°C (3 min) to 300°C at 6°/min	Risk of non-quantitative derivatization[d]
Hydroxy/amino/keto-carboxylic acids	Acetyl, methylates	CP-Sil 19: 70°C (5 min) to 220°C at 5°/min	None
Amino acids	TMS or *t*BDMS	CP-Sil 8: 50°C (5 min) to 150°C at 6°/min, 150–300°C at 12°/min	TMS: multiple derivatives for same compound[b] *t*BDMS: excessive carbon load[c]
Amino acids	TFA, methylates TFA, iso-propylates	CP-Sil 19: 70°C (5 min) to 180°C at 3.5°/min, 180–230°C at 5°/min; 10°/min to 270°C	Poisoning of combustion catalyst[e]
Amino acids	Ethylchloroformates, methylchloroformates	CP-Sil 19: 60°C (3 min) to 100°C at 5°/min, 100–300°C at 10°/min	Risk of non-quantitative derivatization[d]
Amino acids	*N*-Pivaloyl, iso-propylates	CP-Sil 8: 70°C (1 min) to 200°C at 3°/min, 220–300°C at 10°/min	None
Amino acids	*N*-Acetyl, propylates	CP-Sil 19: 70°C (5 min) to 200°C at 4°/min, 200–300°C at 6°/min	None

[a]The content of this column should be read as generalized guidelines providing a starting point for the reader to resolve individual analytical tasks. Information provided is choice of gas chromatography (GC) column (=stationary phase) and temperature programme. CP-Sil is a proprietary name of CHROMPACK International BV (Middelburg, the Netherlands) and has been chosen as an example because it is widely recognized.
[b]Ambiguity of results; a problem for gas chromatography–mass spectrometry (GC–MS) as well as gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS).
[c]Strong dilution of ^{13}C enrichment; this affects only GC–C–IRMS measurements. It is, however, only a problem when the measurement cannot be compared against the background or baseline $\delta^{13}C$ value of an authentic sample.
[d]Potential loss of information (GC–MS and GC–C–IRMS).
[e]A serious problem for GC–C–IRMS; leads ultimately to incomplete combustion and can negatively affect the accuracy and precision of ^{13}C and ^{15}N isotope abundance measurements.
TFA, Trifluoroacetyl; TMS, Trimethylsilyl; *t*BDMS, tert-Butyldimethylsilyl.

apolipoprotein B100 in humans [37•], synthesis rates of α_2-macroglobulin in nephrotic patients [38•] and the protein fractional synthetic rate in skeletal muscle [39•] and plasma albumin [40•].

In the same way, other groups used L-[^{13}C-1]leucine to measure the fractional synthesis rate of mixed muscle protein, myosin heavy chain and actin in human skeletal muscle [41•,42], the metabolism of human apolipoprotein B [43] and protein synthesis in patients suffering from rectal cancer [44,45].

To measure collagen synthesis in adult humans, Rennie *et al.* [3] used L-[^{13}C-1]proline with an enrichment of 30 mol% excess as the marker amino acid. The usefulness of proline as a tracer lies in the phenomenon of post-translational hydroxylation of collagen-bound proline into 4-hydroxyproline and the fact that all protein-bound 4-hydroxyproline is in collagen.

Menand *et al.* [46] described a method to determine low enrichment levels of free L-[^{13}C-1]glutamine in plasma, and Meier-Augenstein *et al.* [12] reported a method to measure [^{13}C-1]-labelled BCAA L-valine, L-leucine and L-isoleucine, as well as their transamination products in human plasma after oral bolus administration. The authors demonstrated that this method could be used to measure whole-body BCAA oxidation in patients suffering from maple syrup urinary disease [12].

High-precision compound-specific isotope analysis of ^{15}N-labelled amino acids

Published work on studies of amino acid and protein turnover using ^{15}N isotopic abundance measurements by GC–C–IRMS are still few and far between. This is quite probably a reflection of the analytical challenges associated with high precision CSIA of ^{15}N isotopic abundance in amino acids. First, the low concentration of nitrogen in amino acids, for amino acids contain two to 11 times more carbon than nitrogen. Second, $\delta^{15}N$ measurements require N_2, which at least doubles the sample size requirement because two molequivalents of amino acids are needed for the production of one molequivalent of N_2. Non-quantitative sample conversion and reduced sensitivity of the IRMS ion source for N_2 (compared with CO_2) increase sample size requirements further, by a factor of five compared with CSIA of ^{13}C-labelled compounds. Finally, even small amounts of atmospheric gas leaks into the GC–C–IRMS instrument result in a high N_2 background level.

However, CSIA of nitrogenous compounds such as amino acids yields new and unexpected insights into the metabolism of organic nitrogen. For example, a study of plasma albumin synthesis using L-[^{13}C-1,^{15}N]alanine and L-[^{13}C-1,^{15}N]leucine showed that both amino acids underwent transamination (and re-amination with nitrogen from the body's nitrogen pool) before their incorporation into plasma protein [3]. The observed enrichment ratios of ^{15}N/^{13}C found in plasma albumin were 1:3.18 and 1:2.29 for alanine and leucine, respectively. A cross-over of ^{13}C could be ruled out because δ^{13}C-values for simultaneously administered L-[^{13}C-1]phenylalanine remained at baseline level throughout the course of the study.

Preston *et al.* [47] measured protein synthesis using [^{15}N]glycine to study the effect of ibuprofen on the plasma concentration of acute-phase proteins in patients with colonic cancer. The authors found that ibuprofen caused a significant reduction of all five acute-phase proteins (fibrinogen, C-reactive protein, caeruloplasmin, α_1-antitrypsin and α_1-acid glycoprotein) after 3 days of oral ibuprofen.

Monitoring δ^{15}N-values of free plasma amino acids from fasting human subjects, Metges and Petzke [48] made the intriguing observation of consistently low levels of ^{15}N abundance ($\delta^{15}N < 0.0‰$ versus air) in the amino acids threonine and phenylalanine. The δ^{15}N values of all the other amino acids were positive ($\delta^{15}N > 0.0$ versus air). In subsequent studies that involved the administration of oral bolus doses of [$^{15}N_2$]urea, Petzke *et al.* [49] found that incorporation of ^{15}N from this source into plasma protein amino acids was consistently low for lysine, histidine, proline and phenylalanine. On the basis of the results of a widened study combining the administration of [$^{15}N_2$]urea with the ingestion of lactic acid bacteria, Petzke *et al.* [50•] hypothesized that these low levels of ^{15}N incorporation suggest only limited or no participation in the transamination reactions of those amino acids.

Conclusion

High-precision CSIA by GC–C–IRMS of organic compounds at natural abundance and low enrichment levels is a powerful tool that provides new insights as well as quantitative information on human metabolism. GC–C–IRMS is a unique tool that can provide answers to biochemical and physiological questions that cannot be obtained by any other analytical instrumentation.

Acknowledgements

The author gratefully acknowledges financial support through the Dietmar–Hopp Foundation for Medical Research and Studies (Walldorf, Germany). Many thanks are due to Dr Helen F. Kemp (University of Dundee) and Professor J. Thomas Brenna (Cornell University) for critical reading of the manuscript.

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Anti-Doping Research Program

Progress Report

March 2004

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Investigators

R. Kazlauskas, G. J. Trout, C. Howe and J. Rogerson (Australian Sports Drug Testing Laboratory)